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(54) Title: HOMOCYSTEINE DESULPHURASE FROM THE PROTOZOAN TRICHOMONAS VAGINALIS

(57) Abstract

The present invention relates to an assay for determining homocysteine, cysteine, O-acetyl-L-serine and/or methionine levels in a biological sample using a enzyme which catalyses the degradation of homocysteine, cysteine, O-acetyl-L-serine and/or methionine, the enzyme being more particularly homocysteine desulphurase, a polynucleotide fragment encoding protozoan homocysteine desulphurase, a recombinant vector comprising such a polynucleotide fragment, transformed cells, the protozoan homocysteine desulphurase polypeptide, and pharmaceutical compositions comprising recombinant homocysteine desulphurase for use in medicine or veterinary medicine.

HOMOCYSTEINE DESULPHURASE FROM THE PROTOZOAN TRICHOMONAS VAGINALIS

The present invention relates to an assay for determing homocysteine, cysteine, O-acetyl-L-serine and/or methionine levels in a biological sample using a enzyme which catalyses the degradation of homocysteine, cysteine, O-acetyl-L-serine and/or methionine, the enzyme being particularly homocysteine desulphurase; a polynucleotide fragment encoding protozoan homocysteine desulphurase, a recombinant vector comprising such a polynucleotide fragment, a host cell containing said polynucleotide fragment or said recombinant vector, the protozoan homocysteine desulphurase polypeptide, and pharmaceutical compositions comprising recombinant homocysteine desulphurase for use in medicine or veterinary medicine.

An elevated level of homocysteine in the blood appears to be an important indicator for many human disease states. Homocysteine is predictive of vascular disease and stroke, Ueland, P.M. (1992) and Kluijtmans L.A.J. et al (1996); is correlated with forms of diabetes and alcoholism, Cravo, M.L. et al (1996); is used to monitor liver and kidney damage, Bostom, A.G. et al (1996) and neural tube defects, Steegers-Theunissen, R.P.N. (1992) and is associated with certain inborn errors of metabolism, Mudd, S.H., (1989).

Homocysteine levels in blood are conventionally determined using high performance liquid chromatography (HPLC) methods, see for example at Poele-Pothoff M.T.B. et al, (1995). However, HPLC methods employ expensive and elaborate machinery, are generally sophisticated and are considered impractical for many routine analyses.

Patent publication WO93/15220 (Cockbain) describes a method for assaying homocysteine in blood using a homocysteine converting enzyme, S-adenosyl homocysteine hydrolase (SAH-hydrolase). SAH-hydrolase catalyses the conversion of homocysteine with a co-substrate, adenosine,

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to S-adenosyl-homocysteine. It is then possible, by determining the amount of adenosine consumed, to make a correlation with the amount of homocysteine consumed. The amount of homocysteine in a sample is then determined from differences in adenosine concentration. However, such an assay requires the use of two initial substrates (homocysteine and adenosine) and two enzymes, making it relatively complex. It also involves determining a decrease in the concentration of adenosine, which may not be satisfactory.

US 4940658 (Allen et al) describes a method for determining sulphydryl amino acids, including homocysteine levels, in samples of body tissues, methods of detecting cobalamin and folic acid deficiency using an assay for total homocysteine levels, and methods for distinguishing cobalamin from folic acid deficiency using an assay for total homocysteine levels in conjunction with an assay for methylmalonic acid. The assays comprise combining a sample with a reference standard comprising a known amount of a sulphydryl amino acid to be assayed, labelled with a suitable marker and measuring the relative amounts of labelled and unlabelled sulphydryl amino acid present for each species with a mass spectrometer. As the amount of labelled species is known, it is therefore possible from calculating the ratio of labelled to unlabelled species to determine the amount of sulphydryl amino acid present in the sample.

US 5438017 describes a gas chromatography/mass spectrometry method for analysis of sulphydryl amino acids in a sample of body fluid. The assay relies on the use of a labelled reference sulphydryl amino acid, similar to that described in US 4940658, but has additional treatment and/or purification steps prior to analysing the sample by gas chromatography/mass spectrometry.

It will be appreciated that similar to HPLC methods, the assays described above which employ gas chromatography/mass spectrometry are generally

sophisticated, use expensive and elaborate machinery and are considered impractical for many routine analyses.

It is an object to the present invention to provide an assay which obviates and/or mitigates at least some of the above disadvantages.

It is a further object of the present invention to provide a recombinant enzyme capable of catalysing the degradation of homocysteine including use in said assay.

The present invention provides a polynucleotide fragment such as a DNA fragment, encoding protozoan homocysteine desulphurase. The invention further provides a recombinant protozoan homocysteine desulphurase polypeptide.

"Homocysteine desulphurase" as used herein refers to an enzyme that is capable of catalysing the degradation of homocysteine to release α-ketobutyrate, hydrogen sulphide, and ammonia.

$$HS-CH_2-CH_2-CH(NH_2)COOH \rightarrow CH_3-CH_2-C(O)COOH + H_2S + NH_3$$
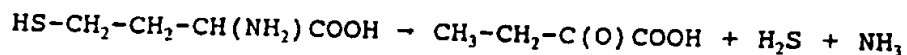
Such an enzyme may also possess an affinity for other substrates such as methionine, cysteine and O-acetyl-L-serine. For example if methionine is used as a substrate, the end products of catabolism by the enzyme are α-ketobutyrate, ammonia and methanethiol.

It should be appreciated that there may be several forms (eg. from different organisms) or isoforms, of "homocysteine desulphurase" and all such forms/isoforms and uses thereof are encompassed herein.

"Polynucleotide fragment" as used herein refers to a chain of nucleotides such as deoxyribose nucleic acid (DNA) sequences and transcription products thereof, such as RNA, capable of giving rise to a homocysteine desulphurase or a physiologically functional derivative thereof. A physiologically functional derivative is one in which the enzyme functionality identifies an enzyme as a homocysteine

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desulphurase as hereinbefore defined. Thus, this term includes double and single stranded DNA, and RNA sequences derived therefrom. The term excludes the whole naturally occurring genome comprising the polynucleotide fragment, as found for example in the protozoon *Trichomonas vaginalis*.

Generally, the polynucleotide will be in substantially isolated form. That is, substantially free of biological material with which the whole genome is normally associated *in vivo*.

In general, the term "polypeptide" refers to a chain or sequence of amino acids displaying a biological activity substantially similar to the biological activity of homocysteine desulphurase and does not refer to a specific length of the product as such. The polypeptide if required, can be modified *in vivo* and/or *in vitro*, for example by glycosylation, amidation, carboxylation, phosphorylation and/or post translational cleavage, thus inter alia, peptides, oligo-peptides, proteins and fusion proteins are encompassed thereby. Naturally the skilled addressee will appreciate that modified polypeptide should retain physiological function i.e. be capable of homocysteine desulphurase activity.

The DNA fragment encoding homocysteine desulphurase can be obtained by utilising a partial homocysteine desulphurase cDNA. The cDNA may be obtained by reverse transcription of messenger RNA followed by amplification typically using polymerase chain reaction (PCR) techniques known in the art, for example, using primers designed against conserved regions of related enzymes such as cystathionine γ-lyase coding sequences e.g. human, rat and/or yeast cystathionine γ-lyase. The amplified fragment containing a portion of the homocysteine desulphurase gene can then be used to clone the entire homocysteine desulphurase gene from a cDNA library comprising such a gene. The cDNA library may be from a protozoan, for example a *Trichomonas vaginalis* cDNA library. Polynucleotide fragments containing homocysteine desulphurase genes

obtained in such a way are depicted in Figures 1 and 2.

The DNA fragment of Figure 1 encodes a gene ctlα (subsequently renamed as mgl1) comprising an open reading frame (ORF) of 396 amino acids hereinafter referred to as CTLα (subsequently renamed as MGL1). The DNA fragment of Figure 2 encodes a gene ctlβ (subsequently renamed as mgl2) comprising an ORF of 398 amino acids hereinafter referred to as CTLβ (subsequently renamed as MGL2). A comparison of the percentage identity (at the amino acid level) of MGL1 and MGL2 as depicted in Figures 1 and 2 with methionine γ-lyase from Pseudomonas putida and cystathionine γ-lyase from yeast and human (EMBL database excession numbers, D30039, P31373 and S52784, respectively) is shown in table 1. Sequence comparison analysis was performed using gap and pileup programs using the GCG Wisconsin package (Devereux, H., Hacberli, P. Smithies, O. (1984) Nucleic Acids Research 12, 387-395).

TABLE 1

	MGL 1	MGL 2	*P.putida* methionine γ-lyase I	yeast cystathionine γ-lyase	human cystathionine γ-lyase
MGL1	-	69%	44%	44%	42%
MGL2		-	45%	43%	43%
P.putida methionine γ-lyase			-	40%	45%
yeast cystathionine γ-lyase				-	52%

Table 1 shows that the putative homocysteine desulphurase enzymes as depicted in Figures 1 and 2 are only 42-45% identical, at the amino acid level, with

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previously sequenced methionine γ-lyase and cystathionine γ- lyases. Thus, although MGL1 and MGL2 may have been cloned using primers designed against conserved regions of cystathionine γ-lyase, they are not substantially similar over the length of the polypeptides.

The present invention also includes polynucleotide fragments having at least 80%, particularly at least 90% and especially at least 95% similarity with the fragment exemplified in Figure 1 and 2. The present invention also includes polypeptide sequences having at least 80%, particularly at least 90% and especially at least 95% similarity with the polypeptide exemplified in Figures 1 and 2. "Similarity" refers to both identical and conservative replacement of nucleotides or amino acids, provided that the enzymic functionality of the homocysteine desulphurase is substantially unimpaired.

The skilled addressee will appreciate that it is possible to genetically manipulate the gene or derivatives thereof, for example, to clone the gene by recombinant DNA techniques generally known in the art and to express the polypeptides encoded thereby in vitro or in vivo. Polynucleotide fragments having the nucleotide sequences depicted in Figures 1 and 2, or derivatives thereof, are preferably used for the expression of homocysteine desulphurase.

It will be understood that for the particular homocysteine desulphurase polypeptides embraced herein, variations (natural or otherwise) can exist. These variations may be demonstrated by (an) amino acid difference(s) in the overall sequence or by deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence. All such derivatives are included within the scope of this invention provided that the derivatives are physiologically functional (ie display homocysteine desulphurase activity as defined herein). For example, for the purpose of the present invention conservative replacements may be made between amino acids,

within the following groups:

    (I)   alanine, serine and threonine;

    (II)   glutamic acid and aspartic acid;

    (III)   arginine and lysine;

    (IV)   asparagine and glutamine;

    (V)   isoleucine, leucine and valine;

    (VI)   phenylalanine, tyrosine and tryptophan.

Moreover, specific replacements of amino acids identified to be within putative functional domains of homocysteine desulphurase may be carried out. For example amino acids identified within a putative substrate binding domain may be replaced with conservative or non-conservative amino acids in order to observe any changes in the enzyme's kinetics such replacements make. Such changes may for example result in an increase in specific activity for homocysteine, or reduce specific activity while decreasing the Km for homocysteine, or increase specific activity for other substrates such as cysteine and O-acetyl-L-serine.

The present inventors have shown that a cysteine residue C113 in MGL1 and C116 in MGL2 plays some part in the homocysteine desulphurase catalytic activity. They have shown that replacement of cysteine 113/116 with glycine still results in a catalytically active homocysteine desulphurase. Although this mutation generally results in an enzyme with reduced specific activity towards all substrates, mutation of cysteine 116 in MGL2 to glycine results in an enzyme with increased specific activity towards cysteine and O-acetyl-L-serine. Moreover mutation of cysteine 116 in MGL2 to glycine results in an enzyme with a lower Km for homocysteine.

In addition the MGL1 and MGL2 sequences have been observed to have a 7 amino acid insertion relative to the cystathionine γ-lyases towards the N-terminus (residues 49-55 in MGL1). Such a region may be suitable for mutation studies.

Mutation studies may allow different homocysteine desulphurases to be produced with differing catalytic activities, which may be suitable for a number of different uses.

Moreover, recombinant DNA technology may be used to prepare nucleic acid sequences encoding these derivatives as outlined above.

As is well known in the art, the degeneracy of the genetic code permits substitution of bases in a codon resulting in a different codon capable of coding for the same amino acid, e.g. the codon for the amino acid glutamic acid is both GAT and GAA. Consequently, it is clear that for the expression of a polypeptide with the amino acid sequence shown in Figures 1 or 2, or a fragment thereof, use can be made of a derivative nucleic acid sequence with such an alternative codon composition different from the nucleic acid sequence shown in Figures 1 or 2.

Furthermore, fragments derived from the homocysteine desulphurase polypeptides or from the amino acid sequences depicted in Figures 1 and 2 which display homocysteine desulphurase activity, or fragments derived from the nucleotide sequence encoding said homocysteine desulphurase polypeptide or derived from the nucleotide sequence depicted in Figures 1 and 2 encoding fragments of said homocysteine desulphurase polypeptides are also included in the present invention.

Naturally the skilled addressee will appreciate that such modifications mentioned hereinabove resulting in enzymically active derivatives of said homocysteine desulphurase polypeptide or gene are encompassed by the present invention. Said homocysteine desulphurase polynucleotide fragments of the present invention are preferably linked to regulatory control sequences. Such control sequences may comprise promoters, operators, inducers, ribosome binding sites, terminators etc. Suitable control sequences for a given host may be selected by those of ordinary skill in the art. Additionally so-

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called "tagging sequences" such as additional amino acids may be added to the N or C terminus of the polypeptide, to give a so-called fusion protein upon expression of the polypeptide.

A polynucleotide fragment according to the present invention can be ligated to any one or more of a variety of expression controlling DNA sequences, resulting in a so-called recombinant DNA molecule. Thus, the present invention also includes an expression vector comprising an expressible nucleic acid molecule. Such recombinant nucleic acid molecules can then be used for the transformation of a suitable host. The expression vectors are preferably hybrid DNA molecules derived from, for example, plasmids, or from nucleic acid sequences derived from bacteriophage or viruses and are termed "vector molecules".

Specific vectors which can be used to clone nucleic acid sequences according to the present invention are known in the art (e.g. Rodriguez, R.L. and D.T. Denhardt, Edit., Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, 1988).

Two specific bacterial expression vectors pQE60 and pQE30 (Qiagen Hilden, Germany) have been used for homocysteine desulphurase expression. The pQE series of expression vectors (e.g. pQE60 and pQE30) encode a 6 histidine tag (6xhis-tag) which enables the purification of fusion protein using metal-chelate affinity chromatography and Fast Protein Liquid Chromatography (FPLC).

The methods used in the construction of a recombinant nucleic acid molecule according to the present invention are known to the skilled addressee and are inter alia set forth in Sambrook, et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, 1989).

The present invention also relates to a transformed cell containing the nucleic acid molecule in expressible form. "Transformation" as used herein, refers to the introduction of a heterologous nucleic acid sequence into

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a host cell, irrespective of the method used, for example direct uptake, transfection or transduction. The heterologous polynucleotide fragment may be maintained through autonomous replication or alternatively, may be integrated into the host genome. The recombinant nucleic acid molecule preferably is provided with an appropriate control sequence compatible with a designated host which can regulate the expression of an inserted polynucleotide fragment e.g. T7 promoter, taq promoter, lac promoter and trp promoter.

Suitable hosts used for the expression of recombinant nucleic acid molecules can be prokaryotic or eukaryotic in origin. The most widely used hosts for expression of recombinant nucleic acid molecules may be selected from bacteria, yeasts, insect cells and mammalian cells. The cloning and expression of recombinant homocysteine desulphurase also facilitates in producing reagents for the production of, for example, probes for *in situ* expression studies, production of anti-homocysteine desulphurase antibodies (particularly monoclonal antibodies) and evaluation of *in vitro* and *in vivo* biological activity of recombinant homocysteine desulphurase.

The present invention further provides recombinant homocysteine desulphurase for the manufacture of reagents for use as prophylactic and/or therapeutic agents. In particular, the present invention provides pharmaceutical compositions comprising the recombinant homocysteine desulphurase together with a pharmaceutically acceptable carrier therefore. Disease states such as cancer may benefit from homocysteine desulphurase therapy and/or prophylactic treatment in a manner similar to that described by Hori, H. et al. (1996) for methionine γ-lyase. Typically homocysteine desulphurase may be used in the development of new antitrichomonal drugs, compounds that may well also have useful activity against other pathogens that contain homocysteine desulphurase and/or methionine γ-lyase. These include both using the recombinant

homocysteine to screen for inhibitors in for example combinatorial libraries and also analysis of the enzyme's structure in order to provide the design of specific inhibitors or pro-drugs.

The present invention provides means with which homocysteine levels may be assayed.

Thus, in a further aspect the present invention provides a method of assaying homocysteine in a sample, comprising the steps of:

a)   contacting the sample with an enzyme capable of degrading homocysteine, and

b)   determining any reaction product(s) formed by enzymic degradation of homocysteine by said enzyme.

Preferably chromatographic separation of homocysteine and/or said reaction product(s) is not carried out.

Preferably the enzyme is homocysteine desulphurase, more preferably is recombinant protozoan homocysteine desulphurase.

Preferably the homocysteine desulphurase is the homocysteine desulphurase according to Figures 1, 2, 6 or 7.

Recombinant protozoan homocysteine desulphurase may also be used to assay other substrates in a sample, including methionine, cysteine and O-acetyl-L-serine.

Generally the biological sample may be a sample of blood, plasma, faeces, saliva, vaginal fluids or urine. Homocysteine may be bound by disulphide linkage to circulating proteins, such as albumin, and homocysteine may also be present in the form of other disulphide derivatives (typically homocysteine-cysteine conjugates). To obtain an estimate of total homocysteine present in the sample it may therefore be desirable to treat the sample with a reducing agent to cleave any disulphide bonds and liberate free homocysteine. Disulphide reduction is reviewed by Jocelyn in Methods of Enzymology 143 : 243-256 (1987)where a wide range of suitable reducing agents are listed. Such suitable

reducing agents are incorporated in the teaching of the present invention.

Conveniently, the end products of the reaction described hereinbefore α-ketobutyrate, hydrogen sulphide and ammonia, may be determined and a variety of suitable methods will be known to the skilled addressee for example, colorimetric, spectrophotometric, electrochemical, fluorimetric or luminescent methods. Preferably the method is sensitive enough to detect concentration of <5 μmol/l homocysteine in a sample.

α-ketobutyrate generated by the degradation of homocysteine may be detected following the method of Soda (Soda, K. (1968) Anal. Biochem. 25: 228-235) using 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH).

An additional method of determining α-ketobutyrate is described by Li, R. + Kenyon, G.L. (1995) A spectrophotometric determination of α-dicarboxyl compounds and its application to the enzymatic formation of α-ketobutyrate. Analytical Biochemistry 230 37-40.

A particularly preferred method of detecting α-keto-butyrate is by adding NADH and lactate dehydrogenase so as to convert the α-ketobutyrate to α-hydroxybutyrate with the generation of NAD^+. The level of NAD^+ can then be measured by a number of methods involving conversion to NADH including spectrophotometrically by absorbance at 340 nm; fluorescently by excitation at 365 nm and emission at 460 nm (Palmer T. (1991) Understanding Enzymes 3rd Edition, Ellis Horwood, London); colorimetrically using tetrazolium salts (Altman, P.F. (1974) Histochemistry 38 p155-171); electrochemically (Morroux J. Elring PJ (1979) Anal Chem 51, 346; Blaedel WJ, Jenkins RA (1975) Anal Chem 47, 1335; Juegfeldt H et al (1981) Anal Chem 53, 1979; Wang J, Lin MS (1987) Electroanal Chem 221, 257); and luminescently (Whitehead TP et al (1979) Clin Chem 25, 1531) As an alternative, pyruvate dehydrogenase may be used in place of lactate dehydrogenase to generate NAD^+ and NAD^+ detected as described above.

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Hydrogen sulphide generated by homocysteine degradation may be determined, for example, by reacting with lead acetate to produce lead sulphide according to the following (stoichiometric) equation.

$$H_2S + Pb(CH_2COOH)_2 \rightarrow PbS + 2CH_3COOH$$
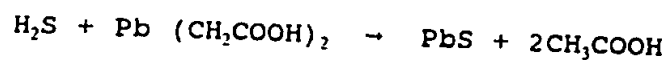
Lead sulphide produced may then be measured spectrophotometrically at a suitable wavelength, such as, A360nm. (Thong K.W + Coombs, G.H. (1985) Homocysteine desulphurase activity in trichomonads. IRCS Medical Science 13 493-494).

Alternatively hydrogen sulphide, may be measured using the methylene blue method as described by Clime, J.D. Limnol, Oceanogr. (1969) 14 : 454-458. Briefly, hydrogen sulphide is reacted with 0.17mM N, N-dimethyl-p-phenylene diamine sulphate in acid and ferric chloride in acid to produce methylene blue which can be detected spectrophotometrically at 650-670nm.

Ammonia generated by the degradation of homocysteine may be reacted with phenol in the presence of hypochlorite to produce indophenol as described by Horn, D.B. + Squire, C.R. (1967), An improved method for the detection of ammonia in blood plasma Clin. Chem. Acta 17 99-105. Indophenol so produced may then be detected spectrophotometrically at a suitable wavelength, for example, 570 nm.

$$NH_3 + OCl + phenol \rightarrow indophenol$$
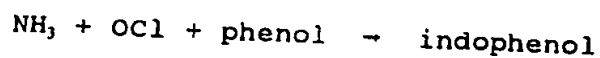
Further methods for detecting ammonia include: enzymatically, using α-ketoglutarate and NAD(P)+ with glutamate dehydrogenase as described by Mondzac A et al (1965) J. Lab. Clin. Med. 66 526; electrochemically using an ammonia electrode as described by Guilbault et al (1985) Anal. Chem. 57 2110; using 2-oxoglutarate and NADH to generate glutamate, water and NAD+ and then measuring NAD+ as described above; and adding silver nitrate to ammonia to

generate a black precipitate.

The recombinant homocysteine desulphurase according to the present invention displays activity towards a number of substrates, in addition to homocysteine, including methionine, cysteine and 0-acetyl-L-serine. It is appreciated therefore that homocysteine desulphurase may be used to assay for methionine, cysteine and/or 0-acetyl-L-serine in a manner similar to that described above.

Furthermore as the recombinant homocysteine desulphurase of the present invention displays activity to a wide range of substrates, the enzyme may be used in the synthesis of unusual amino acids and related molecules

Additionally homocysteine desulphurase may be used to remove homocysteine, methionine and/or cysteine from solutions, for instance from biological media.

Homocysteine desulphurase may also be used to assay for enzymes that catalyse reactions involving homocysteine as either substrate or product (for instance S-adenosylhomocysteine hydrolase). The homocysteine could be assayed in the ways applied to its detection in biological samples. Similarly homocysteine desulphurase may be used to assay enzymes that catalyse reactions involving methionine or cysteine or related compounds as substrates or products. These metabolites could be assayed via their conversion to α-keto acids by homocysteine desulphurase and the measurement of the α-keto acids as described previously.

The assay may also be used to estimate an analyte which is first broken down into homocysteine and then the concentration of the analyte is determined by measuring the concentration of homocysteine. Examples of such analytes include homocystine (where homocystine is converted to homocysteine using DTT) or methionine (which may be enzymatically converted to homocysteine). In both cases the concentration of analyte could thus be determined by measurement of homocysteine.

In a yet further aspect there is provided a kit for diagnostic *in vitro* determination of a homocysteine level in a sample, wherein the kit comprises:

a)   an enzyme capable of degrading homocysteine, and

b)   means for enabling determination of reaction products produced by degradation of homocysteine by the enzyme.

Preferably the enzyme is homocysteine desulphurase, more preferably recombinant protozoan homocysteine desulphurase.

Typically the kit may be in the form of a cuvette based test kit for manual and automated use, microtiter plate test kit or test strip based assay kit.

A particularly preferred kit for diagnostic *in vitro* determination of a homocysteine level in a sample, comprises:

a)   recombinant protozoan homocysteine desulphurase, and

b)   lactate dehydrogenase and NADH for converting α-ketobutyrate, generated by the degradation of homocysteine by said homocysteine desulphurase, into α-hydroxybutyrate with the concomitant release of NAD$^+$, said release of NAD$^+$ being determined by suitable means.

The present invention will now be further described by way of the following non-limiting examples.

Example 1 Design of Primers used to clone *Trichomonas vaginalis* homocysteine desulphurase

Figure 3 shows the multiple protein sequence alignment for cystathionine γ-lyase from human, rat and yeast (Lu et al, 1992, Erickson et al, 1990 and Ono et al, 1992 respectively).   Highlighted are the regions of homology chosen for the design of degenerate oligonucleotides to use as primers in polymerase chain reactions (PCRs).   The first region of homology chosen for the design of the 5' oligonul

ceotide primer was a region in which the sequence between the different cystathionine γ-lyase molecules was highly homologous (V163-N170). The second region chosen for the design of the 3' primer was the pyridoxal 5'-phosphate (PLP) binding domain (A222-G228). The sequences of the degenerate oligonucleotides which were designed based upon these homologous regions is shown in Figure 4. In order to facilitate cloning, restriction sites for the enzymes in *Hind* III and *Xho* I were tagged onto the end of the two degenerate oligonucleotides respectively. The two oligonucleotides were designated Cyst 5' and Cyst 3'. Cyst 5' is 31 nucleotides in length and contains 3 inosines (I) and Cyst 3' is 28 nucleotides in length and contains 2 inosine residues. The inosines were introduced at a number of positions which would have contained four fold degeneracy, in order to reduce the resulting pool of oligonucleotides synthesised. The oligonucleotides were synthesised using an Applied Biosystems DNA synthesiser according to standard protocols.

Example 2   PCRs using the Degenerate Oligonucleotides

RNA Isolation

A clonal cell line (G3) of *T. vaginalis* was grown in modified Diamond's medium as previously described, Lockwood et al. (1984). Cells were harvested (2300g for 15 min at 4°C) in late-phase of growth (1-2 x 10⁶/ml) and washed twice in 0.25M sucrose. DNA was isolated using a Nucleon II kit (Scotlab, Coatbridge, Scotland).

Total RNA was isolated from *T. vaginalis* in a single step using the commercially available TRIZOL (RTM) reagent (available from Gibco, Paisley, Scotland), which is a monophasic solution of phenol and guanidine isothiocyanate, according to the manufacturer's instructions.

Poly[A]⁺ RNA was isolated from total RNA for use as a template for cDNA synthesis. Poly [A]⁺ RNA was isolated using Poly [A]⁺ Quik columns, (available from Stragene, La

Jolla, California, USA) according to the manufacturer's instructions.

One microgram of poly[A]$^+$ RNA from *T. vaginalis* along with reverse transcriptase was used to synthesise first strand *T. vaginalis* cDNA according to procedures as described in Sambrook et al (1989). The cDNA was then used as a template in PCRs with the degenerate oligonucleotides.

Conditions of the PCRs were as follows: initial denaturation at 94°C for 4 minutes followed by 30 amplification cycles consisting of; 94°C for 1 minute, 42°C for 1 minute and 72°C for 1 minute, followed by a final extension cycle at 72°C for 5 minutes. Portions of the reactions were electrophoresed on an agarose gel (1.5%) together with appropriate control reactions. Two PCR products were observed upon staining the agarose gel, one of approximately 200 base pairs (bp) in size (lower band fragment) and a second of approximately 250bp in size (upper band fragment).

Additional identical PCRs were performed in order to obtain enough material for cloning.

Example 3 Cloning of Amplified PCR Products

Material from several PCR reactions (about 1μg of DNA) were combined together and the PCR amplified DNA was phenol/chloroform extracted, ethanol precipitated and resuspended in H$_2$O, to remove contaminating nucleotides and Taq polymerase. The amplified DNA was then restricted to completion with *Hind*III and *Xho*I restriction enzymes (the restriction sites for these had been engineered onto the ends of the amplified DNA by their inclusion at the termini of the Cyst 5' and Cyst 3' degenerate oligonucleotides respectively) to generate "sticky ends" which would facilitate directional cloning. The DNA was purified further by electrophoresis on a 2% TAE agarose gel followed by staining with ethidium bromide and visualisation under long wave UV light. The amplified bands of interest (upper

and lower band products) were excised from the gel using a clean scalpel blade, and the DNA was eluted from the agarose gel slices using commercially available Spin X columns (available from Costar, Cambridge, MA, USA) according to the manufacturer's instructions. The restricted, purified DNA (individual upper band and lower band products and a combination of the two) arising from the PCRs with the degenerate primers, was then combined with pBluescript (available from Stratagene, La Jolla, California, USA) which had also been previously restricted with *Hind* III and *Xho* I, and purified through a 2% TAE agarose gel and eluted using a spin X column, as before. The pBluescript and amplified PCR fragments of approximately 200 and 250bp were ligated in quantities of 200ng insert plus 200ng vector using the Amersham ligation kit (available from Amersham, Little Chalfort, Bucks, UK), according to the manufacturer's instructions.

The ligation reactions were then used to transform ultra-competent XL1 Blue *Escherichia coli* cells (available from Stratagene, La Jolla, California, USA). Approximately 30 to 40ng of ligated DNA was used to transform the competent bacterial cells. Transformation mixes were plated onto LB amp, X-gal, IPTG containing plates, and incubated overnight at 37°C. Plasmid DNA was isolated from white bacterial transformants using the Wizard plasmid mini-prep procedure of Promega (Promega, Madison, Wisconsin, USA) and subjected to selective restriction analysis to ascertain whether cloning of the amplified DNA into pBluescript had been successful.

Transformants containing the cloned PCR products were subjected to sequencing and subsequent analysis in order to determine whether a genuine fragment of a cystathionine γ-lyase homologue had been amplified from the *T. vaginalis* cDNA.

Example 4   Isolation of Full Length *T. vaginalis*
Homocysteine Desulphurase Genes

Two PCR clones (designated cysta 2 and cysta 16) were
used to isolate the corresponding full length genes from a
T. vaginalis λZAP II cDNA library (Mallinson, D.J,
Lockwood, B.C., Coombs, G.H., North, M.J. (1994).
Identification and molecular cloning of four cysteine
proteinase genes from the pathogenic protozoan <u>Trichomonas
vaginalis</u>. J. Gen. Microbiology <u>140</u> 2725-2735). A total
of 100,000 cDNA clones were screened according to the
following procedure. 100,000 bacteriophage were plated in
L-top agarose, along with the host cells *E.coli* XL1 blue
and the bacteriophage plaques were allowed to propagate at
37°C until they were just touching one another in the
bacterial lawn. The bacteriophage plaques were then
transferred by blotting onto a Hybond N nylon filter
(available from Amersham, Little Chalfont, Bucks, UK).

The DNA of the bacteriophage was denatured *in situ* and
subsequently hybridised with either the cysta 2 or cysta 16
200 base pair homocysteine desulphurase fragments,
radioactively labelled by random priming.

Primary screening of the cDNA library, using
conditions of high stringency, 1 hr in 0.1 x SSC/0.1% SDS
at 65°C, revealed that 25 plaques hybridised with the cysta
2 probe, whilst 18 hybridised with the cysta 16 probe.
Autoradiographic film showing the positively hybridising
plaques was aligned with the plates containing the
bacteriophage in order to allow plugs of agarose containing
the positive bacteriophage to be removed. The plugs were
placed into SM buffer and a trace of chloroform at 4°C and
the phage allowed to defuse out overnight.

A second round of bacteriophage purification was
carried out in order to identify individual plaques which
hybridised under high stringency conditions with either the
cysta 2 or cysta 16, 200 base pair radioactive probe.

Example 5   Analysis of Cysta 2 and Cysta 16 hybridising Clones

Two λ clones which hybridised with the cysta 2 probe and five λ clones which hybridised with the cysta 16 probe were rescued directly into pBluescript using the F1 helper bacteriophage mechanism (according to the manufacturers instructions, Stratagene, La Jolla, California, USA, protocols).   The rescued plasmids were subsequently analysed by restriction analysis to determine the sizes of cloned insert DNA.

As a result of the restriction analysis of the plasmids isolated with the cysta 2 or cysta 16 probes, two clones, one a cysta 2 hybridiser (*ctlα*, subsequently renamed as *mgl1*) and the other a cysta 16 hybridiser (*ctlβ* subsequently renamed as *mgl2*), were chosen to be fully sequenced.   These two clones were chosen as they had the largest inserts (approximately 1.2 to 1.3 kilobases in size) and were thought to be of sufficient size to encode a full length copy of the a homocysteine desulphurase gene from *T. vaginalis*.

Example 6   Sequencing of Two *T. vaginalis* Homocysteine Desulphurase Genes.

Restriction mapping of each of the two *T. vaginalis* homocysteine desulphurase genes was carried out in order to allow subcloning of smaller fragments that would assist in obtaining the full nucleotide sequence of each gene.

Sequencing of the two clones and their respective subclones was achieved using Sequenase(RTM) quick Denature Plasmid sequencing kit (available from Amersham, Little Chalfront, Bucks, UK) with T7 and T3 primers (available from Stratagene, LA Jolla, California, USA) according to the manufacturer's instructions.   The complete nucleotide sequence and predicted amino acid sequence of the first clone (*mgl1*) and the complete nucleotide sequence and

predicted amino acid sequence of the second clone (*mgl2*) was determined.

In order to obtain the 5' untranslated regions (UTRs) of *mgl1* and *mgl2* and to confirm the start codons, 5' Reverse Transcriptase Rapid Amplification of cDNA ends (RT-RACE) was performed using the commercially available 5' RACE kit (available from Gibco, Paisley, Scotland) according to the manufactures instructions. Both the *mgl1* and *mgl2* RACE products which were obtained were approximately 250 base pairs in size. The *mgl1* and *mgl2* RACE products were cloned directly into a pTAg vector of the ligATor kit (available from R & D Systems, Minneapolis, USA), according to the manufacturer's instructions. This system exploits the feature that PCRs performed in the presence of Taq polymerase have a 5' adenosine overhang tagged onto the ends of any amplified fragments. The system facilitates the cloning of PCR products into a vector which has a complementary thymidine residue overhang.

Restriction analysis of the transformants revealed that the 5' RACE products had been cloned into the pTAg vector. Sequencing of the RACE clones was carried out on both strands using commercially available -20 primer and M13 reverse primer.

Sequencing of the 5' RACE product of *mgl1* revealed that the 5' UTR is very short (13 nucleotides long), but confirmed the start ATG codon identified from the cDNA sequence.

Two independent 5' RACE products were obtained for *mgl2*, both clones possessed the ATG start codon which was absent from the copy of the gene isolated from the cDNA library. The longer *mgl2* RACE clone identified a short 5' UTR region of some 14 nucleotides in length.

Both the *mgl1* and *mgl2* 5' UTRs are shown together with the respective complete cDNA sequence and predicted amino acid sequences of *mgl1* and *mgl2* in Figures 1 and 2. Pileup analysis (see Table 1) revealed the relatively low level of

sequence identity of the putative MGL1 and MGL2 to previously sequenced cystathionine γ-lyases and that it was unlikely that a *T. vaginalis* cystathionine γ-lyases had been cloned. This was confirmed by the finding that the cloned gene products do not have cystathionine γ-lyase activity (see Table 2).

Example 7 Cloning and Expression of histidine-tagged homocysteine desulphurase fusion protein

The QIAexpress system (Qiagen, La Jolla, California, USA) was used for the expression and purification of MGL1 and MGL2 polypeptides. The *mgl1* and *mgl2* genes were cloned into a pQE vector which encodes a 6-Histidine tag at the N or C terminus of the expressed protein. The 6-Histidine tagged protein is then affinity purified using a Ni^{2+}-NTA resin (see QIAexpress handbook for details).

Cloning of *mgl1*

The *mgl* cDNA clone was maintained in the pasmid pBluescript. Preliminary sequence analysis indicated that the *T.vaginalis mgl1* cDNA had been cloned into pBluescript in the reverse orientation to what was expected, therefore direct subcloning of *mgl1* from pBluescript to a pQE vector was not possible. In order to overcome the problems of the orientation of *mgl1* cDNA in pBluescript, a PCR cloning strategy was adopted to enable the cDNA to be cloned into an appropriate pQE vector.

Oligonucleotide primers were designed to the 5' and 3' ends of the *mgl1* cDNA which included the restriction endonuclease sites *Nco*I and *Bgl*II, respectively. Through the PCR amplification process these two restriction sites were engineered on to the ends of the *mgl1* DNA, their presence facilitating cloning of DNA into *Nco*I and *Bgl*II restricted pQE vector. The nucleotide sequence of the two primers are shown in Figure 5.

pQE 60, a type ATG construct was chosen as the vector into which the *mgl1* DNA was to be cloned. The ATG construct allows the expressed protein to start with the authentic ATG codon. The *mgl1* cDNA encoded a start methionine and was therefore considered suitable for cloning into this particular pQE vector (see QIA express handbook for details).

pBluescript containing *mgl1* cDNA was linearised using *Bam*HI and the linearised DNA was used as the template for PCRs along with the two oligonucleotides outlined in Figure 5. The components of the PCR mix are outlined below.

$1\mu l$ ($10ng/\mu l$) pBluescript/*mgl1* *Bam*HI linearised template
$5\mu l$ ($100ng/\mu l$) 5' *Nco*I primer
$5\mu l$ ($100ng/\mu l$) 3' *Bgl*II primer
$5\mu l$ 10x pfu buffer (Stratagene, La Jolla, California, USA)
$2.5\mu l$ 5mM each of dATP, dGTP, dCTP, dTTP
$1\mu l$ pfu polymerase (a proofreading version of Taq available from Stratagene, La Jolla, California, USA)
$30.5\mu l$ water

Amplification of the *mgl1* DNA was performed using the conditions outlined below.

94°C for 5 minutes followed by 30 cycles of
94°C for 1 minute,
42°C for 1 minute, and
72°C for 1 minute and finally a single extension reaction of
72°C for 5 minutes

After amplification by PCR, contaminating nucleotides and polymerase were removed from the *mgl1* DNA using Magic PCR Wizard preps (Promega, Madison, Wisconsin, USA) according to the manufacturers instructions. The cleaned up *mgl1* DNA which now possessed an *Nco*I site at its 5' end

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and a *Bgl*II site at its 3' end was restricted with these two enzymes as was the pQE60 vector. The restricted pQE vector and *mgl1* DNA were ligated and intact vector containing insert was transformed into M15[pREP4] cells, see QIAexpress handbook for details. pQE 60 plasmid containing *mgl1* DNA was then used for test expression of recombinant protein according to the QIAexpress handbook.

Cloning of *mgl2*

mgl2 cDNA contained within pBluescript was subcloned directly into pQE30. pBluescript containing *mgl2* DNA was restricted with *Bam*HI and *Xho*I and this restricted insert was ligated with pQE30 vector that had been restricted with *Bam*HI and *Sal*I. Intact pQE30 vector and insert was transformed into M15 pREP4 cells. pQE 30 vector containing *mgl2* DNA was then used for test expression of recombinant protein according to the QIAexpress handbook.

The pQE plasmids containing either *mgl1* or *mgl2* were transformed into <u>E.coli</u> strain M15[pREP4]
and single colonies obtained. A single colony was inoculated into 20ml LB-both containing 100ug/ml ampicillin and 25ug/ml kanamycin grown overnight at 37°C.

1 litre of LB-broth was then inoculated with the entire overnight culture and the culture grown at 37°C until A_{750} reached 0.8 (approximately 2-3 hours). IPTG was then added to a final concentration of 1mM and growth was allowed to continue at 37°C for a further 2 1/2 hours.

The cells were harvested by centrifugation and frozen at -70C.

Expressed protein was purified by FPLC using a metal chelating resin, such as Ni-NTA superflow, according to protocol 5 of the Qiagen QIAexpress protocols.

Protein-containing fractions obtained by FPLC were analysed by SDS-PAGE and those with recombinant homocysteine desulphurase were combined and dialysed overnight against sonication buffer (50mM Na-phosphate pH8.0, 300mM NaCl) containing 10% glycerol before storage

at -20°C.

Example 8 Modified procedure for the purification of recombinant *T. vaginalis* homocysteine desulphurase

The procedures detailed below encompass growth of bacteria, expression of recombinant enzyme and FPLC/Ni-NTA purification. Details of all buffers, media etc are given in the appendix. Additional details of vector, bacterial host strain and protocols can be found with reference to the QIAGEN protein expression handbook.

Day 1
Streak out 5 µl of the supplied glycerol stock of M15[pREP4] cells (containing pQE30/*T. vaginalis* cDNA) onto Luria-Bertani (LB) agar plates containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml). Grow up overnight @ 37°C. [Colonies on LB plates can be stored at 4 °C for up to 2 weeks].

Day 2
In a 500ml flask inoculate 50 ml of LB broth containing ampicillin and kanamycin (final concentrations as above) with a single colony. Grow up overnight at 37°C with shaking - 200rpm in an orbital shaker.

Day 3
In a 2 litre flask, inoculate 400 ml of fresh LB broth plus ampicillin and kanamycin with the 50 ml overnight culture.
- Grow the culture for 1.75 h with shaking (200rpm) at 37°C. Induce the cells to express homocysteine desulphurase by addition of sterile IPTG (to give a final concentration of 0.2mM) and grow for a further 2.25h with shaking at 37°C.
- Pellet the cells by centrifugation at 8000g at 4°C for 10-15 min.
- Resuspend the pellet in 5 ml of sonication buffer and transfer to a 15 ml Falcon tube, add pyridoxal 5′ phosphate

(PLP) to a final concentration of 20 μM. Freeze the resuspended cells at -70°C until required for purification. N.B. To check that expression has worked, 200 μl samples of bacterial culture are removed a) just before addition of IPTG and b) after the addition of IPTG, at the end of the 2.25 h induction period. The cells are pelleted (13000 rpm / 5 min), resuspended in 80 μl of Laemmli's sample buffer, boiled for 5 min, and a 10 μl aliquot run on a 12.5 % SDS-PAGE gel to confirm expression of homocysteine desulphurase after induction by IPTG. [Alternatively a larger volume of uninduced and induced cells (1 ml) may be sampled, lysed by sonication and a homocysteine desulphurase activity assay carried out].

Equilibrate Ni-NTA resin column overnight with <u>sonication buffer</u>. Typically 8ml packed volume of resin/column may be used.

Day 4 <u>Affinity purification of His-tagged enzyme on Ni-NTA resin by FPLC.</u>

1. Remove frozen cells from -70°C freezer, thaw by placing the tube in a beaker of cold water.

2. Lyse the cells by sonication.

3. Transfer the sonicated material to a 50 ml centrifuge tube and centrifuge at 10,000g for 30 min at 4°C.
It is useful to check that the bacteria have been lysed adequately, especially when first using a sonicator, by comparing pellet and supernatant fractions by SDS-PAGE. Homocysteine desulphurase is highly soluble and 95% should be found in the soluble fraction.

4. After centrifugation, the supernatant [which contains the soluble enzyme] ($\sim$ 5-6ml total vol.) is filtered through a 0.22 μm Millipore filter directly into a Luer-

lock syringe attached to the priming nozzle (injection port) of the FPLC and the purification started.

Note : The purification procedure outlined was performed on a Waters FPLC system including a Waters 600S controller and Waters 626 pump. The basic steps are :
sample application (the sample is automatically drawn from the syringe onto the column when the priming nozzle is turned to 'injection' mode). The enzyme can actually be seen to bind to the NI-NTA resin, as a very bright yellow band.

1.   short wash with sonication buffer
2.   longer wash with wash buffer
3.   elution of enzyme using a linear gradient 0-500 mM imidazole [100% wash buffer to 100% elution buffer (inc. 500 mM imidazole)]

Note : for full details of FPLC running conditions (flow rates, durations of washes, gradients etc.) see appendix.

5. The protein concentration in the column outflow is monitored continuously by a UV detector set at 280 nm, and fractions are collected throughout the procedure.

6.   Fractions containing recombinant enzyme (easily identified by their bright yellow-green colour) are pooled and dialysed against 1 litre of dialysis buffer overnight (with several changes if necessary) at 4°C (to remove the imidazole).

7.   A small sample (~ 50 μl) of the dialysed enzyme preparation is taken for determination of the protein content using the BCA procedure (Pierce Chemical Company BCA [Bicinchoninic acid] reagent kit). The remainder of the preparation is combined 1:1 with stabilisation buffer, and stored in 1 ml aliquots @ -20°C.

APPENDIX

Reagents and buffers required.

<u>Luria- Bertani Medium (LB medium)</u>

For 1 litre :
Dissolve the following in 950 ml H_2O :

bacto-tryptone	10 g
bacto yeast extract	5 g
NaCl	10 g

Adjust pH to 7.0 (if necessary). Make to 1 l with dist. H_2O. Sterilise by autoclaving for 20 min. @ 15 lb/sq. in. (For LB agar, include 15 g of bacto agar per litre).

<u>Antibiotics</u>

Ampicillin (Sigma A-9518)
(100 mg/ml stock in distilled H_2O) - sterilised by filtration through a 0.2 μm Millipore filter, stored as 1 ml aliquots @ -20°C.

Kanamycin (Sigma K-4000)
(25 mg/ml stock in dist. H_2O) - sterilised and stored as above.

<u>IPTG (isopropyl-β-D-thiogalactopyranoside; Gibco BRL 15529-019)</u>

1 M stock (in dist. H_2O) - filter sterilised (0.2 μm filter), stored in 1ml aliquots @ -20°C

<u>Pyridoxal 5'-phosphate (PLP; Sigma P-9255)</u>

1 mM stock in dist. H_2O. Make up fresh each time (for addition to resuspended cells).

<u>Ni-NTA Superflow resin (QIAGEN 30430)</u>
<u>Sonication buffer: 50 mM sodium phosphate buffer, pH 8.0,</u>
<u>300 mM NaCl</u>

1 M Na_2HPO_4 : 46.6 ml
1 M NaH_2PO_4 :   3.4 ml
17.53g NaCl
Make to 1 litre with dist. H_2O.

<u>Wash buffer: 50 mM sodium phosphate buffer, pH 6.0, 300 mM</u>
<u>NaCl, 10 % glycerol</u>

1 M Na_2HPO_4 :   6 ml
1 M NaH_2PO_4 : 44 ml
17.53g NaCl
100 ml glycerol
Make to 1 l with dist. H_2O

<u>Elution buffer: wash buffer containing 500 mM imidazole</u>

Dissolve 17.02g imidazole in 500 ml wash buffer.
(Imidazole - Sigma I-0125)
<u>Dialysis buffer: 100 mM sodium phosphate buffer, pH 6.5,</u>
<u>300 mM, 20% glycerol, 20 μM PLP, 15 μM dithiothreitol</u>

1 M Na_2HPO_4 : 30.35 ml
1 M NaH_2PO_4 : 69.65 ml
17.53g NaCl
200 ml glycerol
2 ml of 10 mM PLP stock
15 μl of 1 M dithiothreitol stock (dithiothreitol - Sigma
D-9779)

<u>Stabilisation buffer: 100 mM sodium phosphate buffer pH</u>
<u>6.5, 80% glycerol, 40 μM PLP, 30 μM DTT</u>

For 10 ml :   1.96 ml of 100 mM sodium phosphate buffer, pH 6.5 8 ml glycerol, 40 μl of 10 mM PLP, 3 μl of 100 mM DTT

Sodium azide (Sigma S-2002) : 0.05% solution

- pump onto Ni-NTA column after use to prevent bacterial growth (store column in azide @ 4°C between purifications).

Make 10 % (w/v) stock in dist. H_2O (store @ 4°C). Dilute to give 0.05 % working concentration.

All buffers used on FPLC should be degassed prior to use Degassing achieved by filtration of buffer through a 0.2 μm filter using a Millipore vacuum filter unit.

Homocysteine desulphurase activity assay used to monitor purification

Homocysteine   →   2-ketobutyrate + NH_3 + H_2S

The assay measures the production of H_2S; H_2S is 'trapped' by lead acetate forming colloidal lead sulphide (a deep brown coloured compound) which has maximal absorbance at 360 nm.

Reagents

Assay buffer : 100 mM imidazole buffer, pH 6.5

D,L-homocysteine (Sigma H-4628); stock solution 100 mM (made up in assay buffer). Final concentration in assay = 40 mM (400 μl of stock)

Lead acetate (BDH 10142); stock solution 3.3 mM (made up in dist. H_2O). Final concentration in assay = 0.33 mM (100 μl of stock)

Recombinant enzyme: as a start point, use 10 μl of a 100 x dilution of the pure enzyme prep. (dilute enzyme prep. in 100 mM sodium phosphate buffer, pH 6.5)

Final volume of assay mixture is made up to 1.0 ml with assay buffer.

Run control assay mixtures: 1) minus enzyme, 2) minus substrate.

Calculation of enzyme activity

Use molar extinction coefficient for lead sulphide of 5205 M^{-1} cm^{-1}.

$$\frac{\text{change in abs}_{(expll)} - \text{change in abs.}_{(control)} \times 10^6}{\text{time x protein concentation x 5205 } M^{-1} \text{ } cm^{-1} \times 10^3}$$

= μ moles min^{-1} mg $prot^{-1}$

Details of FPLC programme for purification of recombinant *T. Vaginalis* MGL2

GRADIENT

	TIME	FLOW	%A	%B	%C
1.	(Sample applied 0 - 30 min)	0.2	100	0	0
2.	30	0.5	100	0	0
3.	90	0.5	0	100	0
4.	150	0.5	0	100	0
5.	250	0.5	0	0	100

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Buffers   :   A =   Sonication buffer

                 B = Wash buffer

                 C = Elution buffer

EXAMPLE 9   Homocysteine assays

ASSAY I

        Homocysteine levels were measured using recombinant homocysteine desulphurase prepared according to the previous examples in 66mM sodium phosphate buffer pH 7.5, 0.33 mM lead acetate.

        Homocysteine desulphurase catalyses the conversation of homocysteine to α-ketobutyrate, ammonia and hydrogen sulphide. The hydrogen sulphide reacts with lead acetate to produce lead sulphide which can be detected spectrophotometrically at 360 nm (molar extinction coefficient = $5205M^{-1}cm^{-1}$).

assay reagents

0.1 M sodium phosphate	0.5 ml
1mM lead acetate	0.5 ml
homocysteine 100 μM to 10 mM (33μM - 3.3 mM final concentration)	0.49 ml
homocysteine desulphurase (6μg/ml) -added last	10 μl

The assay was incubated at 20°C for 0 to 120 minutes to allow the colour to develop. The homocysteine levels were then determined by measuring the absorbance at 360 nm.

Results

homocysteine concentrations	change in absorbance (40 min)
3.3mM	1.066
333μM	0.790
33μM	0.06

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ASSAY II

Assay Principle

Dithiothreitol (DTT) is initially used to break homocystine down into homocysteine and to release protein bound homocyst(e)ine. The homocysteine is then broken down into α-ketobutyrate, NH_3 and H_2S by the action of homocysteine desulphurase. A specific lactate dehydrogenase isoenzyme is then utilised to convert α-ketobutyrate into α-hydroxybutyrate with the concomitant release of NAD^+. After removal of any NADH by lowering of the pH using HCl the NAD is fed into a cycling mechanism involving ethanol, alcohol dehydrogenase, diaphorase and tetrazolium salts to generate a coloured product which can be photometrically measured. The increase in colour corresponds to the concentration of homocysteine in the sample.

Performance of the assay

Step 1: Mix 100 ul of sample (e.g citrated plasma) with 500 ul 0.1mol/l HEPES, 0.1 mmol/l NADH, 20,000 μmoles/min/l homocysteine desulphurase, 50,000 U/l Lactate dehydrogenase and 0.05 mol/l dithiothreitol, pH 8.0 into a cuvette. Incubate at 37°C for 20 min.

Step 2: Add 500 ul 1 mol/l HCl, 0.55% (v/v) Nonidet P40, 1×10^{-4} mol/l nitroblue tetrazolium (NBT). Incubate at 37°C for 5 min.

Step 3: Add 500 ul tris (hydroxymethylaminomethane) (TRIS), 1 mol/l ethanol followed by 50 ul 20,000 U/l alcohol dehydrogenase, 1000 U/l diaphorase.

Measure the increase in absorbance at 527 nm for 5 minutes after the addition of the reagent containing alcohol dehydrogenase.

Assay performance

i) standard curve

A typical standard curve is shown in the table below:

Homocysteine concentration (µmol/l)	Delta Abs. 527 nm/10 min
0	0.385
10	0.495
20	0.580
30	0.670
40	0.770
50	0.850

Standard curve was generated by spiking homocysteine into serum. The background signal is in part caused by endogenous levels of homocysteine

ii) Sensitivity

It is clearly possible to detect concentrations of < 5 µmol/l homocysteine.

iii) Recovery

Homocysteine was spiked into plasma and the recovery determined:

Homocysteine added (µmol/l)	Homocysteine recovered (µmol/l)	% recovery
25	20.7	83
50	53.2	106
75	65.5	87
100	95.7	96

iv) Linearity

The following table illustrates the linearity of the response:

Amount of saline added (%)	Measured delta Abs. 527 nm/10 min	Theoretical delta Abs. 527 nm/10 min	Signal as percent of expected signal
0	0.52	0.52	100%
10	0.45	0.47	95.7%
20	0.41	0.42	97.6%
30	0.35	0.37	94.6%
40	0.3	0.31	96.8%
50	0.22	0.26	84.6%
100	0	0	-

A patient sample (plasma) was diluted using the above amounts of saline and the measured signal compared to the theoretical signal.

v) Cross reactivity

The following table illustrates the cross reactivity of the assay with methionine and cysteine:

Concentration of analyte (μmoles/l)	Delta Abs. 527 nm/10min		
	Homocysteine	Methionine	Cysteine
0	0.52	0.52	0.52
25	-	0.51	0.48
50	-	0.52	0.5
100	-	0.49	0.51
200	1.1	0.5	0.49

Homocysteine, methionine and cysteine were spiked into plasma and the signal measured. No cross reactivity with either cysteine or methionine was observed up to levels of 200μmols/l.

Example 10   Site-directed mutagenesis of recombinant MGL1 and MGL2

The production of mutated rMGL1 (C113G) and rMGL2 (C116G) was achieved using the PCR-based QuikChange ™ Site-Directed Mutagenesis kit (Stratagene). Double-stranded *mgl1* and *mgl2* cDNAs (in either p-Bluescript or pQE30/60 expression vectors) were used as templates. Mutagenesis was performed using a pair of oligonucleotide primers complementary to opposite strands of the cDNA clones, each containing a point mutation to convert the respective cysteine codons (TGC) to glycine codons (GGC), as follows: 1) 5'- TGCCTTTATGGCGGCACACATGCTCTCT -3' ; 2) 5'-AAGAGAGCATGTGTGCCGCCATAAAGG -3'. Following PCR-mediated amplification of mutated cDNAs, the original template cDNAs/vectors were selectively digested using the *Dpn*-I endonuclease. cDNA clones containing the desired mutations were identified by sequencing on both strands using gene specific oligonucleotide primers. cDNAs mutated in p-Bluescript were subcloned into pQE vectors prior to expression. Production and purification of recombinant mutated MGL1 and MGL2 was performed as described previously.

Example 11   Enzymatic studies of recombinant MGL1, and MGL2, and mutated MGL1 (C113G) and MGL2 (C116G)

Methionine γ-lyase previously purified from *T. vaginalis* has activity towards a number of substrates, including methionine, homocysteine and S-adenosylmethionine, but has no activity towards cystathionine (Lockwood and Coombs, 1991). In order to assess the similarity between MGL1 and MGL2 and purified native methionine γ-lyase, the enzyme activities of the

recombinant enzymes were analysed (Table 2). rMGL1 and rMGL2 were found to have very high activity towards homocysteine, and also to catabolise methionine, cysteine and O-acetylserine rapidly. The two recombinant enzymes were unable to utilise cystathionine as a substrate. The kinetic parameters of the two recombinant proteins were also determined for homocysteine and cysteine (Table 3). The apparent K_m of rMGL1 for the two substrates was higher than those for rMGL2, the largest difference being for methionine.

Following production and purification of mutated rMGL1 (C113G) and rMGL2 (C116G), their enzymatic activities were compared with those of the corresponding wild-type enzymes (Table 2). Under optimal conditions the activities of rMGL1 (C113G) towards all substrates were considerably lower than those of wild-type rMGL1. rMGL2 (C116G) also had lower activities towards homocysteine and methionine than wild-type rMGL2, but surprisingly the activity of the mutated enzyme towards cysteine and O-acetyl-L-serine was increased. Neither of the mutated enzymes exhibited activity towards cystathionine.

Comparative kinetic analyses of the mutated and wild-type enzymes with respect to the catabolism of homocysteine and cysteine were performed (Table 3). The slightly higher K_m and markedly lower V_{max} values of rMGL1 (C113G) compared with those of wild-type rMGL1 suggest reduced substrate binding efficiency of this mutated enzyme. In contrast, the apparent K_m of rMGL2 (C116G) for cysteine was considerably lower than that of rMGL2, and this correlates with the enhanced activity (higher V_{max}) of the mutated enzyme towards this substrate. Unexpectedly the K_m of rMGL2 (C116G) for homocysteine was also much reduced relative to that of the wild-type enzyme, despite the significantly lower V_{max} of the mutated enzyme towards this substrate.

Table 2. Comparison of enzymatic activities of mutated and wild-type recombinant proteins

Substrate	rMGL1	rMGL1(C113G)	mutant/wildtype(%)
Homocysteine	370 ± 11 (8)	34.5 ± 3.2 (14)	9.3
Methionine	10.4 ± 0.31 (4)	0.79 ± 0.17 (8)	7.6
Cysteine	6.02 ± 0.63 (8)	2.33 ± 0.35 (8)	38.7
O-acetyl-L-serine	3.74 ± 0.1 (4)	1.83 ± 0.12 (8)	48.9
Cystathionine	N.D (4)	N.D (8)	-

Substrate	rMGL2	MGL2(C116G)	mutant/wildtype (%)
Homocysteine	128 ± 22 (14)	27.0 ± 5.8 (19)	21.2
Methionine	0.67 ± 0.18 (11)	0.15 ± 0.05 (14)	22.4
Cysteine	1.06 ± 0.42 (16)	2.31 ± 0.71 (17)	217.9
O-acetyl-L-serine	1.51 ± 0.49 (12)	2.15 ± 0.17 (14)	142.4
Cystathionine	N.D (12)	N.D (8)	-

Activities (in μmol min^{-1} mg protein^{-1}) are means ± S.D. from the number of experiments given in parentheses. Activity towards homocysteine and cysteine was assayed by monitoring hydrogen sulphide production using the standard procedure; activity towards the other substrates was measured via the standard α-keto acid production assay. N.D., activity not detectable (<0.04 μmol min^{-1} mg protein^{-1}).

Table 3 Kinetic parameters of wild type and mutated rMGL1 and rMGL2 with respect to catabolism of homocysteine and cysteine

	Homocysteine		Cysteine	
	K_M[1]	V_{max}[2]	K_m[1]	V_{max}[2]
rMGL1	12.2	256	8.5	14.9
rMGL1 (C113G)	15.2	42	9.7	4.6
rMGL2	37.7	132	22.3	2.4
rMGL2 (C116G)	6.2	53	3.6	4.8

At least 10 different substrate concentrations were used, with at least 3 replicate assays.

[1]mM, [2]μmol min^{-1} mg protein[-1].

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- CLAIMS -

1. A method of assaying homocysteine in a sample, comprising the steps of:
    a) contacting the sample with an enzyme capable of degrading homocysteine, and
    b) determining any reaction product(s) formed by enzyme degradation of homocysteine by said enzyme.

2. A method of assaying homocysteine according to claim 1 wherein the enzyme is homocysteine desulphurase.

3. A method of assaying homocysteine according to claim 2 wherein the homocysteine desulphurase is a recombinant protozoan homocysteine desulphurase or functionally active derivative thereof displaying homocysteine desulphurase activity.

4. A method of assaying homocysteine according to claim 3 wherein the recombinant protozoan homocysteine desulphurase is a homocysteine desulphurase substantially as shown in Figures 1,2,6 or 7.

5. A method of assaying homocysteine according to any one of claims 1 to 4 wherein one of said reaction product(s) is α-ketobutyrate and a level of α-ketobutyrate is determined.

6. A method of assaying homocysteine according to any one of claims 1 to 4 wherein one of said reaction product(s) is hydrogen sulphide and a level of hydrogen sulphide is determined.

7. A method of assaying homocysteine according to any one of claims 1 to 4 wherein one of said reaction(s) is ammonia and a level of ammonia is determined.

8.   A method of assaying homocysteine according to claim
     5 wherein the level of -ketobutyrate is determined by
     reacting the α-ketobutyrate with NADH and lactate
     dehydrogenase or pyruvate dehydrogenase so as to
     convert the α-ketobutyrate to a α-hydroxybutyrate with
     the generation of NAD$^+$ and determining a level of
     NAD$^+$.

9.   A method of assaying homocysteine according to any one
     of claims 1 to 8 wherein said method is sensitive
     enough to detect a concentration of <5 μmol/l
     homocysteine.

10. A method of assaying an analyte degradable to
     homocysteine, which comprises first degrading the
     analyte to homocysteine and then estimating the
     homocysteine produced by the method of any preceding
     claim.

11. A method according to claim 10 wherein the analyte is
     homocystine or methionine.

12. A method of assaying methionine, cysteine or 0-acetyl-
     L-serine in a sample comprising the steps of:
     a)   contacting the sample with recombinant protozoan
          homocysteine desulphurase or functionally active
          derivative thereof capable of degrading
          methionine, cysteine or 0-acetyl-L-serine, and
     b)   determining any reaction product(s) formed by
          enzyme degradation of methionine, cysteine or 0-
          acetyl-L-serine.

13. A kit for diagnostic *in vitro* determination of a
     homocysteine level in a sample, wherein the kit
     comprises:
     a)   an enzyme capable of degrading homocysteine, and
     b)   means for enabling determination of reaction

products produced by degradation of homocysteine by the enzyme.

14. A kit for diagnostic *in vitro* determination of a homocysteine level in a sample according to claim 13 wherein the enzyme is homocysteine desulphurase.

15. A kit for diagnostic *in vitro* determination of a homocysteine level in a sample according to claim 14 wherein the homocysteine desulphurase is a recombinant *protozoan* homocysteine desulphurase or functionally active derivative thereof displaying homocysteine desulphurase activity.

16. A kit for diagnostic *in vitro* determination of a homocysteine level in a sample according to claim 15 wherein the recombinant protozoan homocysteine desulphurase is a homocysteine desulphurase substantially as shown in Figures 1,2,6 or 7.

17. A polynucleotide fragment encoding a protozoan homocysteine desulphurase.

18. A polynucleotide fragment according to claim 17 wherein the polynucleotide fragment is a deoxyribose nucleic acid (DNA) fragment.

19. A polynucloetide fragment according to either of claims 17 or 18 characterised in that said polynucleotide fragment encodes a polypeptide having an amino acid sequence substantially as shown in Figures 1,2,6 or 7 or a functionally active derivative thereof.

20. A polynucleotide fragment according to claim 17 characterised in that it is a polynucleotide fragment which is substantially the same as the polynucleotide

45

fragment shown in Figures 1,2,6 or 7 or a functionally active derivative thereof.

21. A recombinant nucleic acid molecule comprising a polynucleotide fragment according to any one of claims 17 to 20.

22. A recombinant nucleic acid molecule according to claim 21 characterised in that the recombinant nucleic acid molecule comprises regulatory control sequences operably linked to said polynucleotide fragment for controlling expression of said polynucleotide fragment.

23. A recombinant nucleic acid molecule according to either of claims 21 and 22 wherein the recombinant nucleic acid molecule is a plasmid.

24. A recombinant nucleic acid molecule according to either of claims 21 and 22 wherein the recombinant nucleic acid molecule is derived from a viral vector.

25. A prokaryotic or eukaryotic host cell transformed by a polynucleotide fragment or recombinant molecule according to any one of claims 17 to 24.

26. A recombinant *protozoan* homocysteine desulphurase polypeptide or functionally active derivative thereof displaying homocysteine desulphurase activity.

27. A recombinant *protozoan* homocysteine desulphurase polypeptide as shown in Figures 1,2,6 or 7 or functionally active derivative thereof.

28. An antibody immuno-reactive with a polypeptide or fragment according to either of claims 26 and 27.

29. A polynucleotide fragment according to any one of claims 17 to 20 for use in therapy.

30. A recombinant nucleic acid molecule according to any one of claims 21 to 24 for use in therapy.

31. A recombinant protozoan homocysteine desulphurase or functionally active derivative thereof according to either of claims 26 and 27 for use in therapy.

32. Use of a recombinant polypeptide or functionally active derivative thereof according to either of claims 26 and 27 in the manufacture of a medicament for use in therapy.

33. Use of a recombinant polypeptide or functionally active derivative thereof according to either of claims 26 and 27 in the manufacture of a medicament for use in cancer therapy.

34. A pharmaceutical composition comprising a polynucleotide fragment according to any of claims 17 to 20 together with a pharmaceutically acceptable carrier.

35. A pharmaceutical composition comprising a polypeptide or derivative thereof according to claims 26 and 27 together with a pharmaceutically acceptable carrier.

36. Use of a recombinant polypeptide or functionally active derivative thereof according to either of claims 26 and 27 to screen for inhibitors thereof.

37. Use of a recombinant polypeptide or functionally active derivative thereof according to either of claims 26 and 27 to remove homocysteine, methionine

and/or cysteine from a sample.

38. Use of a recombinant polypeptide or functionally
     active derivative thereof according to either of
     claims 26 and 27 to determine the presence of enzymes
     and catalyse reactions involving homocysteine,
     methionine or cysteine as either substrate or product.

FIGURE 1

```
   1  ATTTTTAGACAACATGTCTCACGAGAGAATGACCCCAGCAACAGCATGCATCCATGCTAA   60
   1               M  S  H  E  R  M  T  P  A  T  A  C  I  H  A  N   20

  61  TCCACAGAAGGATCAGTTTGGAGCAGCCATCCCACCAATCTACCAAACATCAACATTCGT  120
  21   P  Q  K  D  Q  F  G  A  A  I  P  P  I  Y  Q  T  S  T  F  V   40

 121  TTTCGATAACTGCCAACAGGGTGGAAACAGATTCGCTGGTCAGGAATCCGGCTACATCTA  180
  41   F  D  N  C  Q  Q  G  G  N  R  F  A  G  Q  E  S  G  Y  I  Y   60

 181  CACACGTCTCGGCAACCCAACAGTTTCAAACCTCGAAGGCAAGATCGCCTTCCTCGAGAA  240
  61   T  R  L  G  N  P  T  V  S  N  L  E  G  K  I  A  F  L  E  K   80

 241  AACAGAAGCATGCGTTGCCACATCTTCTGGCATGGGTGCCATTGCTGCTACAGTTTTGAC  300
  81   T  E  A  C  V  A  T  S  S  G  M  G  A  I  A  A  T  V  L  T  100

 301  AATCCTCAAGGCCGGAGATCACTTAATCTCCGATGAGTGCCTTTATGGCTGCACACATGC  360
 101   I  L  K  A  G  D  H  L  I  S  D  E  C  L  Y  G  C  T  H  A  120

 361  TCTCTTTGAGCACGCATTGACAAAGTTCGGCATCCAGGTCGACTTCATCAACACAGCCAT  420
 121   L  F  E  H  A  L  T  K  F  G  I  Q  V  D  F  I  N  T  A  I  140

 421  CCCAGGCGAGGTCAAGAAGCACATGAAGCCAAACACAAAGATTGTCTATTTCGAGACACC  480
 141   P  G  E  V  K  K  H  M  K  P  N  T  K  I  V  Y  F  E  T  P  160

 481  AGCCAACCCAACACTCAAGATCATCGACATGGAGCGCGTCTGCAAGGACGCCCACAGCCA  540
 161   A  N  P  T  L  K  I  I  D  M  E  R  V  C  K  D  A  H  S  Q  180

 541  GGAGGGCGTCTTAGTTATCGCCGATAACACATTCTGCTCACCAATGATCACAAACCCAGT  600
 181   E  G  V  L  V  I  A  D  N  T  F  C  S  P  M  I  T  N  P  V  200

 601  CGACTTTGGCGTCGATGTTGTTGTCCACTCTGCAACAAAGTACATCAACGGCCACACAGA  660
 201   D  F  G  V  D  V  V  V  H  S  A  T  K  Y  I  N  G  H  T  D  220

 661  TGTCGTCGCTGGCCTTATCTGTGGCAAGGCTGACCTCCTTCAACAGATTCGTATGGTTGG  720
 221   V  V  A  G  L  I  C  G  K  A  D  L  L  Q  Q  I  R  M  V  G  240

 721  TATCAAGGATATCACAGGATCTGTTATCAGCCCACACGACGCTTGGCTCATCACACGTGG  780
 241   I  K  D  I  T  G  S  V  I  S  P  H  D  A  W  L  I  T  R  G  260

 781  CCTCTCAACACTCAACATCAGAATGAAGGCTGAGAGCGAGAACGCCATGAAGGTCGCTGA  840
 261   L  S  T  L  N  I  R  M  K  A  E  S  E  N  A  M  K  V  A  E  280

 841  GTACCTCAAATCTCACCCAGCCGTTGAGAAGGTTTACTACCCAGGCTTCGAGGACCACGA  900
 281   Y  L  K  S  H  P  A  V  E  K  V  Y  Y  P  G  F  E  D  H  E  300

 901  GGGCCACGATATCGCTAAGAAGCAGATGAGAATGTCGGGTTCAATGATCACATTCATCCT  960
 301   G  H  D  I  A  K  K  Q  M  R  M  S  G  S  M  I  T  F  I  L  320

 961  CAAGTCCGGCTTCGAAGGCGCTAAGAAGCTCCTCGACAACCTCAAGCTTATCACACTTGC 1020
 321   K  S  G  F  E  G  A  K  K  L  L  D  N  L  K  L  I  T  L  A  340

1021  AGTTTCCCTTGGTGGCTGCGAGTCCCTCATCCAGCACCCAGCTTCAATGACTCACGCTGT 1080
 341   V  S  L  G  G  C  E  S  L  I  Q  H  P  A  S  M  T  H  A  V  360

1081  CGTTCCAAAGGAGGAGCGTGAGGCCGCTGGTATTACAGATGGCATGATCCGCCTTTCTGT 1140
 361   V  P  K  E  E  R  E  A  A  G  I  T  D  G  M  I  R  L  S  V  380

1141  CGGTATTGAAGATGCCGACGAACTCATCGCTGATTTCAAACAGGGCCTTGACGCTCTTTT 1200
 381   G  I  E  D  A  D  E  L  I  A  D  F  K  Q  G  L  D  A  L  L  400

1201  ATAAACTCTACTTAGTTTCTTGACTTTAATTAAAAAAAAAAAAAAAAAAAA 1248
```

FIGURE 2

```
   1  GACTTTATATAAAAGATGAGTGGCCACGCTATCGACCCAACACATACAGACACACTTTCC    60
   1                 M  S  G  H  A  I  D  P  T  H  T  D  T  L  S     20

  61  ATCCACGCCAACCCACAGAAGGATCAGTTCGGTGCTATTGTTGCTCCAATCTACCAAACA   120
  21  I  H  A  N  P  Q  K  D  Q  F  G  A  I  V  A  P  I  Y  Q  T     40

 121  TCCACCTTCCTCTTCGACAACTGCGACCAGGGTGGTGCTCGTTTCGGTGGCAAGGAAGCC   180
  41  S  T  F  L  F  D  N  C  D  Q  G  G  A  R  F  G  G  K  E  A     60

 181  GGTTACATGTACACACGTATCGGTAACCCAACAAACTCCGCACTCGAAGGCAAGATCGCC   240
  61  G  Y  M  Y  T  R  I  G  N  P  T  N  S  A  L  E  G  K  I  A     80

 241  AAGCTCGAACACGCTGAGGCATGCGCTGCCACAGCTTCTGGCATGGGTGCTATTGCTGCT   300
  81  K  L  E  H  A  E  A  C  A  A  T  A  S  G  M  G  A  I  A  A    100

 301  TCTGTCTGGACATTCCTCAAGGCCGGTGATCACCTTATCTCCGACGATTGCCTTTATGGC   360
 101  S  V  W  T  F  L  K  A  G  D  H  L  I  S  D  D  C  L  Y  G    120

 361  TGCACACACGCCCTCTTCGAGCATCAGCTCCGCAAGTTCGGCGTTGAAGTTGATTTCATC   420
 121  C  T  H  A  L  F  E  H  Q  L  R  K  F  G  V  E  V  D  F  I    140

 421  GACATGGCTGTCCCAGGAAACATTGAGAAGCACTTGAAGCCAAACACAAGAATCGTCTAC   480
 141  D  M  A  V  P  G  N  I  E  K  H  L  K  P  N  T  R  I  V  Y    160

 481  TTCGAAACACCAGCTAACCCAACATTAAAGGTTATCGACATCGAAGACGCCGTCAAGCAG   540
 161  F  E  T  P  A  N  P  T  L  K  V  I  D  I  E  D  A  V  K  Q    180

 541  GCCAGAAAGCAGAAGGATATCCTCGTTATCGTTGATAACACCTTCGCTTCACCAATTCTT   600
 181  A  R  K  Q  K  D  I  L  V  I  V  D  N  T  F  A  S  P  I  L    200

 601  ACAAACCCACTCGACCTCGGTGTTGATATCGTCGTTCACTCCGCTACTAAGTACATCAAT   660
 201  T  N  P  L  D  L  G  V  D  I  V  V  H  S  A  T  K  Y  I  N    220

 661  GGCCACACCGATGTTGTCGCCGGCCTTGTCTGCTCAAGAGCTGACATCATCGCTAAGGTC   720
 221  G  H  T  D  V  V  A  G  L  V  C  S  R  A  D  I  I  A  K  V    240

 721  AAGTCCCAGGGTATCAAGGATATCACAGGCGCCATCATTTCCCCACACGACGCTTGGCTC   780
 241  K  S  Q  G  I  K  D  I  T  G  A  I  I  S  P  H  D  A  W  L    260

 781  ATCACAAGAGGCACACTTACACTCGATATGCGTGTCAAGCGCGCTGCCGAGAACGCTCAG   840
 261  I  T  R  G  T  L  T  L  D  M  R  V  K  R  A  A  E  N  A  Q    280

 841  AAGGTCGCTGAATTCCTCCATGAGCACAAGGCCGTCAAGAAGGTCTACTACCCAGGCCTT   900
 281  K  V  A  E  F  L  H  E  H  K  A  V  K  K  V  Y  Y  P  G  L    300

 901  CCAGACCATCCAGGCCACGAAATCGCCAAGAAGCAGATGAAGATGTTCGGCTCTATGATC   960
 301  P  D  H  P  G  H  E  I  A  K  K  Q  M  K  M  F  G  S  M  I    320

 961  GCATTCGATGTCGACGGATTAGAGAAGGCCAAGAAAGTCCTTGACAACTGCCACGTTGTT  1020
 321  A  F  D  V  D  G  L  E  K  A  K  K  V  L  D  N  C  H  V  V    340

1021  TCTCTCGCCGTTTCCCTCGGTGGTCCAGAATCCCTCATCCAGCACCCAGCTTCAATGACA  1080
 341  S  L  A  V  S  L  G  G  P  E  S  L  I  Q  H  P  A  S  M  T    360

1081  CACGCTGGTGTTCCAAAGGAGGAACGCGAGGCTGCTGGCCTAACAGATAACCTCATCCGC  1140
 361  H  A  G  V  P  K  E  E  R  E  A  A  G  L  T  D  N  L  I  R    380

1141  CTCTCTGTTGGCTGTGAGAACGTTCAGGATATCATCGACGACCTCAAGCAGGCTCTCGAC  1200
 381  L  S  V  G  C  E  N  V  Q  D  I  I  D  D  L  K  Q  A  L  D    400

1201  TTAGTCCTCTAAATTTAACTTTCGAATTTCAGTAATAAAATCCTAGATATCTTCCCCCCC  1260
 401  L  V  L                                                       420

1261  CAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  1305
```

Figure 3

```
         1                                                      50
cyhuman  MQEKDASSQG FLPHFQHFAT QAIHVGQDPE QWTSRAVVPP ISLSTTF...
  cyrat  .......... .......... .......... .....CCGAA HLLATTF...
cyyeast  .........M TLQESDKFAT KAIHAGEHVD VHGS..VIEP ISLSTTF...

         51                                                    100
cyhuman  ..KQGAP..G QHSG.FEYSR SGNPTRNCLE KAVAALDGAK YCLAFASGLA
  cyrat  ..KQDSP..G QSSG.FVYSR SGNPTRNCLE KAVAALDGAK HCLTFARGLA
cyyeast  ..KQSSP..A NPIGTYEYSR SQNPNRENLE RAVAALENAQ YGLAFSSGSA

         101                                                   150
cyhuman  A.TVTITHLL KAGDQIICMD DVYGGTNRYF RQVASEFGLK ISFVDCSKIK
  cyrat  A.TTTITHLL KAGDEVICMD EVYGGTNRYF RRVASEFGLK ISFVDCSKTK
cyyeast  T.TATILQSL PQGSHAVSIG DVYGGTHRYF TKVANAHGVE TSFTN.DLLN

         151                                                   200
cyhuman  LLEAAITPET KI|VWIETPTN| PTQKVIDIEG CAHIVHKHG. ..DIILVVDN
  cyrat  LLEAAITPQT KI|VWIETPTN| PTLKLADIKA CAQIVHKHK. ..DIILVVDN
cyyeast  DLPQLIKENT KI|VWIETPTN| PTLKVTDIQK VADLIKKHAA GQDVILVVDN

         201                                                   250
cyhuman  TFMSPYFQRP LALGADISMY S|ATKYMNG|HS DVVMGLVSVN CESLHN.RLR
  cyrat  TFMSAYFQRP LALGADICMC S|ATKYMNG|HS DVVMGLVSVN SDDLNE.RLR
cyyeast  TFLSPYISNP LNFGADIVVH S|ATKYING|HS DVVLGVLATN NKPLYE.RLQ

         251                                                   300
cyhuman  FLQNSLGAVP SPIDCYLCNR GLKTLHVRME KHFKNGMAVA QFLESN.PWV
  cyrat  FLQNSLGAVP SPFDCYLCCR GLKHCRSGWR NTFQDGMAVA RFLESN.PRV
cyyeast  FLQNAIGAIP SPFDAWLTHR GLKTLHLRVR QAALSANKIA EFLAADKENV

         301                                                   350
cyhuman  EKVIYPGLPS HPQHELVKRQ CTGC..TGMV TFYIKGTLQH AEIFLKNLKL
  cyrat  EKVIYPGLPS HPQHELAKRS ARAC..PGMV SFYIKGTLQH AQVFLKNIKL
cyyeast  VAVNYPGLKT HPNYDVVLKQ HRDALGGGMI SFRIKGGAEA ASKFASSTRL

         351                                                   400
cyhuman  FTLAESLGGF ESLAELPAIM THASVLKNDR DVLGISDTLI RLSVGLEDEE
  cyrat  FALAESLGGY ESLAELPAIM THASVPEKDR ATLGISDTLI RLSVGLEDEK
cyyeast  FTLAESLGGI ESLLEVPAVM THGGIPKEAR EASGVFDDLV RISVGIEDTD

         401                 422
cyhuman  DLLEDLDQAL KAAHPPSGIH S*
  cyrat  DLLEDLGQAL KAAHP*.... ..
cyyeast  DLLEDIKQAL KQATN*.... ..
```

Figure 4

Cyst 5'


```
5' GCAAGCTTGTITGGATTGAGACICCIACGAA 3'
      HindIII           C           A
                        A           T
                                    C
```


Cyst 3'


```
5' GCCTCGAGCCGTTIATGTACTTIGTAGC 3'
      XhoI           A  T      G
                               C
                               T
```


Figure 5

5' NcoI primer

```
5'                     3'
CGCCATGGCTCACGAGAGAATGAC
   NcoI
```

3' BglII primer

```
5'                        3'
GCAGATCTTAAAAGAGCGTCAAGGCCC
   BglII
```

FIGURE 6

The nucleotide sequence of mutated *mgl*1 and the MGL1 (C113G) amino acid sequence are identical to that of *mgl*1/MGL1 with the following change (the change being shown underlined):

```
332   GATGAGGGCCTTTAT      346
111   D   E   G   L   Y    115
```

FIGURE 7

The nucleotide sequence of mutated *mgl*2 and the MGL2 (C116G) amino acid sequence are identical to that of *mgl*2/MGL2 with the following change (the change being shown underlined):

```
343   GACGATGGCCTTTAT      357
      D   D   G   L   Y    119
```

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N15/60   C12N9/88   C12Q1/527   C07K16/40   A61K38/51
        C12N15/62   C12P13/12   C12Q1/68   //(C12N9/88,C12R1:90)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   C12Q   A61K   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 15220 A (COCKBAIN JULIAN R M ;AXIS RESEARCH (NO)) 5 August 1993 cited in the application	1,13
Y	see the whole document	2-12, 14-38

Y	THONG K-W ET AL: "TRICHOMONAS-SPP HOMOCYSTEINE DESULFURASE AND SERINE SULFHYDRASE ACTIVITIES." EXP PARASITOL 63 (2). 1987. 143-151. CODEN: EXPAAA ISSN: 0014-4894, XP002049548 see the whole document	1-38

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
9 December 1997	23/12/1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040. Tx. 31 651 epo nl. Fax: (+31–70) 340–3016	Hix, R

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	LOCKWOOD B C ET AL: "PURIFICATION AND CHARACTERIZATION OF METHIONINE GAMMA LYASE FROM TRICHOMONAS-VAGINALIS." BIOCHEM J 279 (3). 1991. 675-682. CODEN: BIJOAK ISSN: 0306-3275, XP002049549 see the whole document ---	1-38
Y	P.F. ERICKSON ET AL.: "Sequence of cDNA for rat cystathionine gamma-lyase and comparison of deduced amino acid sequence with related Escherichia coli enzymes" THE BIOCHEMICAL JOURNAL, vol. 269, 1990, pages 335-340, XP002049550 cited in the application see the whole document ---	1-38
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A	K-W. THONG ET AL.: "Homocysteine desulphurase activity in trichomonads." IRCS MED. SCI. , vol. 13, no. 6, 1985, pages 493-494, XP002049553 see the whole document ---	1-38
A	US 4 940 658 A (ALLEN ROBERT H ET AL) 10 July 1990 cited in the application see the whole document -----	1-38

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